Crossed Immunoelectrophoresis of Human Platelet Membranes

EFFECT OF CHARGE ON ASSOCIATION AND DISSOCIATION OF THE GLYCOPROTEIN GPIIb-GPIIIa MEMBRANE COMPLEX*

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The mechanism of association of the human platelet membrane GPIIb-GPIIIa-Ca2+ complex was studied by treating solubilized membranes with various enzymes and cationic peptides and by studying the binding of 45Ca2+ and 125I-fibrinogen before and after dissociation with EGTA and association with Ca2+. Neuraminidase shifted the complex cathodally (presumably due to cleavage of negatively charged domains), whereas trypsin had no such effect. The EGTA-dissociated complex was almost completely reassociated with neuraminidase or the cationic peptide, tetrasine. The monoclonal antibody 10E5, which specifically binds to the Ca2+-associated complex (not to its dissociated components), also bound to the neuraminidase-associated complex. Thus, Ca2+ is not necessary for the association of the complex. Neuraminidase treatment of washed intact platelets resulted in a cathodal shift of the membrane Triton X-100-extracted associated complex with no effect on its ability to dissociate in the presence of EGTA. Neuraminidase treatment of ADP-perturbed washed platelets also resulted in a cathodal shift of the associated complex; however, dissociation with EGTA was inhibited. Thus, critical neuraminidase-sensitive components of the complex (sialic acid residues) are not exposed on the surface of the platelet membrane of resting platelets, but do become accessible following platelet stimulation with ADP or membrane solubilization with Triton X-100.

45Ca2+ bound to the associated complex, to GPIIb of the dissociated complex (not to GPIIIa), to the Ca2+-reassociated complex, and to the neuraminidase-associated complex which had been dissociated with EGTA. Thus, neuraminidase-sensitive components of the solubilized membrane are not required for Ca2+ binding. 125I-fibrinogen bound to the associated complex (not the dissociated complex), to the Ca2+-reassociated complex, and to the neuraminidase-reassociated complex which had been dissociated with EGTA. Thus, Ca2+ is not necessary for 125I-fibrinogen binding to the major antigen complex.

Recent developments in platelet membrane biochemistry have revealed the presence of a platelet membrane glycoprotein complex (1, 2) of glycoproteins GPIIb-GPIIIa held together by Ca2+ and dissociated with divalent cation chelating agents (3-7). This complex, which appears as the major antigen following crossed immunoelectrophoresis of platelet membranes, ranges from absent to diminished in Glanzmann's thrombasthenia (1, 2, 5), a bleeding disorder in which platelets do not bind fibrinogen nor aggregate with physiologic platelet agonists (8-10). The GPIIb-GPIIIa-Ca2+ complex appears to be the receptor for platelet fibrinogen (11-14), which becomes operative following activation of the platelet membrane with ADP (8, 9, 15-18). Monoclonal antibody studies have suggested that GPIIb-GPIIIa are associated in the intact, nonstimulated platelet (12). Since platelets under basal conditions do not bind fibrinogen, and the GPIIb-GPIIIa-Ca2+ complex appears to be the fibrinogen receptor, it has been inferred that the GPIIb-GPIIIa-Ca2+ complex may be "loosely" associated under basal conditions and undergoes further conformation following platelet activation. Since platelet fibrinogen binding requires Ca2+ as well as platelet membrane activation, it has been assumed that Ca2+ is required for binding of fibrinogen to the GPIIb-GPIIIa complex.

We have performed crossed immunoelectrophoresis on platelet membranes in order to determine the mechanism of glycoprotein complex association, Ca2+ binding, and fibrinogen binding. This was investigated by studying the effect of neuraminidase, as well as cationic peptides, on the reassociation of the EGTA-dissociated GPIIb-GPIIIa-Ca2+ complex, and by studying the effect of neuraminidase on binding of Ca2+ and fibrinogen to the complex.

EXPERIMENTAL PROCEDURES

Methods

Preparation of Platelet Membranes—Platelet-rich plasma was obtained from The New York Blood Center and centrifuged at 2,800 × g for 15 min at 4 °C. The platelet pellet was washed in a human Ringer's solution (19) containing 2 mM EDTA, 10 mM benzamidine, 100 μg/ml soybean trypsin inhibitor, and 1% ammonium oxalate, pH 7.1, as described previously (2, 5), plus 0.1 mM phenylmethylsulfonyl fluoride and 0.2 mM leupeptin. Platelet membranes were prepared as described previously (2, 5) by four cycles of freezing and thawing, followed by sonication and centrifugation on a cushion of 30% sucrose dissolved in 0.01 M Tris buffer, pH 7.4, containing 0.15 M NaCl, and the aforementioned protease inhibitors. The membranes were washed once in 0.15 M NaCl containing protease inhibitors, centrifuged at 100,000 × g for 1 h at 4 °C, and solubilized.

Solubilization of Platelet Membranes with Triton X-100—Platelet membranes were adjusted to approximately 5 mg/ml protein after extraction with 1% (v/v) Triton X-100 containing 0.07 M Tris, 0.22 M sodium barbiturate buffer, pH 8.6, and protease inhibitors at room temperature.

The abbreviations used are: EGTA, [ethylenebis(oxyethylene-nitrito)]tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CIE, crossed immunoelectrophoresis.
Effect on GPIIb-GPIIIa Complex

Effect of Charge on GPIIb-GPIIIa Complex

Materials

All materials were obtained as previously described (2, 5). Trypsin-L-1-tosylamido-2-phenyl ethyl chloromethyl ketone (200 units/ml, bovine pancreas) was obtained from Worthington. Tetralysine and arginylarginine were obtained from Vega Biochemicals, Tucson, AZ. Neuraminidase (type VI, Clostridium perfringens) mucin, leupeptin, phenylmethylsulfonyl fluoride, and EGTA were obtained from Sigma. 4Ca²⁺ (74 mBq/ml) was obtained from Amersham Corp. Highly purified 125I-fibrinogen (1.3 x 10⁶ cpm/µg), labeled with IODO-BEADS, was kindly supplied by Dr. Marjorie Zucker of New York University Medical School. The monoclonal antibody (Fab')2 fragment) was kindly supplied by Dr. Barry Coller of State University Medical Center, Stony Brook, NY. It was isotopically labeled with 125I by the IODO-GEN (Pierce) method, according to the procedure recommended in their Technical Bulletin, to a specific activity of 6 x 10⁶ cpm/µg. Na¹⁰⁵ (100 mCi/ml) was obtained from Amersham Corp.

RESULTS

Effect of Trypsin or Neuraminidase on the Intact Dissociated and Reassociated Major Antigen GPIIb-GPIIIa-Ca²⁺ Complex—Initial experiments were performed to probe the antigenic determinant requirements for the reassociation of the dissociated GPIIb-GPIIIa complex by digesting the Triton-solubilized membranes with elastase, trypsin, or neuraminidase. Elastase partially associated the dissociated GPIIb-GPIIIa major antigen complex and resulted in a cathodal shift of the complex (23). Trypsin had no effect on the dissociated complex. However, neuraminidase did associate the dissociated complex (see below).

Neuraminidase—Fig. 1, a–d demonstrates the effect of neuraminidase treatment. Neuraminidase resulted in a cathodal shift of the major antigen GPIIb-GPIIIa complex, with a shortening and widening of the major antigen peak (Fig. 1d). As previously reported (5), EGTA dissociated the major antigen IIb-IIIa complex into GPIIb and GPIIIa (Fig. 1b). Ca²⁺ reassociated this dissociated complex (Fig. 1c). A striking

Purification of Glycoproteins GPIIb-GPIIIa—Preparative gel electrophoresis was performed with Triton-solubilized platelet membranes on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (20), employing slab gels, 10 x 14 x 1/⁴ mm. These preparations gave single bands with appropriate molecular weights, when run on SDS-PAGE stained with Coomassie Brilliant Blue, and single immunoprecipitate arcs, with appropriate electrophoretic mobilities, when run on CIE against rabbit anti-platelet membrane antibody.

Incubation of the Platelet Membrane Major Antigen (GPIIb-GPIIIa-Ca²⁺ Complex)—Triton-solubilized platelet membranes were incubated at pH 8.6 in the presence of varying concentrations of EGTA for 5 min at room temperature (these conditions were shown to be sufficient for dissociation of the complex), followed by the addition of Trit/tarbit/Triton buffer, pH 8.6, or the addition of this buffer plus CaCl₂, or the addition of this buffer plus trypsin, neuraminidase, or cationic peptide for varying periods of time. Digestion with trypsin was performed in the absence of protease inhibitors. Neuraminidase digestion was performed in the presence of protease inhibitors plus Trit/tarbit/Triton buffer cited above. Protease activity was not detectable, employing a [H]caseinolytic assay (addition of 50,000 cpm of ³H]casein plus 0.1 unit of neuraminidase per 100-µl sample (21)). Neuraminidase activity was operated at 60% efficiency as determined by digestion of mucin (0–600 µg/250 µl) with 0.1 unit of neuraminidase in 0.2 M Tris maleate buffer, pH 5.6, employing the thiorbarbituric assay (22).

Crossed Immunelectrophoresis of Platelet Membranes—This was performed with rabbit anti-platelet membrane antibody as previously described (2, 5), employing 50 µg of platelet membrane and 100–150 µl/µl anti-platelet membrane antibody raised in various rabbits.

 Autoradiography of ⁴Ca²⁺, ¹²⁵I-Fibrinogen, and ¹²⁵I-Labeled 10E5 Antibody Binding to the Major Antigen Complex—Slides were washed and blotted in the 154 M, 20 mM Tris-HCl, 0.05% bovine serum albumin, pH 7.4 (2, 5), and then incubated in the same buffer containing ⁴Ca²⁺ (1–2 µC/ml), ¹²⁵I-fibrinogen (1 x 10⁶ cpm/ml), or ¹²⁵I-10E5 (1 x 10⁶ cpm/ml) overnight at room temperature. The slides were then rinsed, washed, blotted with paper towels, and washed and blotted three times in the same buffer without isotope or albumin. The blotted slides were dried in an oven at 40 C, and exposed to Kodak film at -60 C for various time intervals (1–10 days). The slides were then stained with Coomassie Brilliant Blue as described previously (2, 5) and compared to their autoradiograms.

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Effect of Charge on GPIIb-GPIIIa Complex

14268

Effect of Charge on GPIIb-GPIIIa Complex

FIG. 2. Binding of 125I-labeled monoclonal antibody 10E5 to the associated, EGTA-dissociated, neuraminidase-reassociated, and neuraminidase-treated major antigen complex of the Triton-solubilized membranes. a, 50 μg of control platelet membranes; b, membranes incubated with 4 mM EGTA for 5 min, followed by 25 min with buffer; c, membranes incubated with 0.5 units of neuraminidase for 25 min, followed by buffer for 5 min; d, membranes incubated with 4 mM EGTA for 5 min, followed by 0.5 units of neuraminidase/50 μg of membrane for 25 min. The washed, blotted CIE slides were then incubated with 125I-10E5 (100,000 cpm/ml) overnight, followed by washing, blotting, and drying prior to radioautography. Autoradiograms of slides e–h refer to the respective slides a–d, which have been stained with Coomassie Brilliant Blue.

FIG. 3. Effect of neuraminidase treatment of intact washed platelets on the CIE pattern of the EGTA-dissociated and Ca2+-reassociated major antigen complex of the Triton-solubilized platelet membrane. Washed platelets were treated with 1 unit/ml neuraminidase in a 10-ml volume (4 × 10^9 platelets/ml) for 15 min at 37°C in human Ringer solution containing protease inhibitors at pH 7.1. a, 50 μg of control membranes prepared from intact platelets incubated with buffer (instead of neuraminidase); b, membranes (prepared from intact platelets incubated with neuraminidase) incubated with 4 mM EGTA for 5 min, followed by buffer for 20 min; c, similar membranes as in b, incubated with 4 mM EGTA for 5 min, followed by 3 units of neuraminidase/50 μg of membrane for 20 min; d, similar membranes as in b, incubated with 4 mM EGTA for 5 min, followed by 8 mM CaCl2 for 20 min.

This observation was the reassociation of the EGTA-dissociated complex (Fig. 1b) by neuraminidase treatment alone (Fig. 1c). This could be achieved with as little as 0.01 unit of neuraminidase/50 μg of membrane for 20 min at room temperature (data not shown). Neuraminidase had its effect on GPIIb rather than GPIIIa, since the cathodal GPIIb arc disappeared after neuraminidase treatment, to complex with the GPIIIa arc, which did not change its electrophoretic position, but stained more intensely. In other studies, purified GPIIb moved cathodally when treated with 0.2 unit of neuraminidase.
Effect of Charge on GPIIb-GPIIIa Complex

Fig. 4. Effect of ADP and neuraminidase treatment of intact washed platelets on the CIE pattern of the EGTA-dissociated complex of the Triton-solubilized membrane. Unstirred washed platelets were incubated with 20 μM ADP with or without 1 unit/ml neuraminidase in a 10-ml volume (1 × 10⁹ platelets/ml) for 30 min at room temperature in the presence of human Ringer solution, pH 7.1, containing protease inhibitors. a, Triton-solubilized membrane preparation from original platelets treated with ADP, incubated in CIE buffer for 5 min at room temperature prior to CIE; b, same membrane preparation as a, but exposed to 3 mM EGTA at room temperature for 5 min, prior to CIE; c, Triton-solubilized membrane preparation from original platelets treated with ADP plus neuraminidase, incubated in CIE buffer prior to CIE; d, same preparation as c, but exposed to 3 mM EGTA as in b. The multiple arcs in the IIb region (above and below the arrow) are variably seen and probably reflect microheterogeneity of the GPIIb arc.

Fig. 5. Binding of ⁴⁵Ca²⁺ to the associated, EGTA-dissociated, neuraminidase-reassociated, and neuraminidase-treated major antigen complex of the Triton-solubilized platelet membrane. a, 50 μg of control platelet membranes; b, membranes incubated with 0.5 mM EGTA for 5 min; c, membranes incubated with 0.5 mM EGTA for 5 min, followed by 0.1 unit of neuraminidase/50 μg of membrane for 20 min; d, membranes incubated with 0.1 unit of neuraminidase for 20 min, followed by buffer for 5 min. The washed, blotted CIE slides were then incubated with ⁴⁵Ca⁺ for 15 h, followed by washing, bloting, and drying prior to radioautography. Autoradiograms of slides e-h refer to their respective slides a-d, which have been stained with Coomassie Brilliant Blue. (Note absence of radioactivity for IIIa on autoradiogram f.)

for 20 min (4 experiments), whereas purified GPIIIa was unaffected by neuraminidase treatment (data not shown).

¹²⁵I-Labeled Monoclonal Antibody 10E5 Binding to Neuraminidase-treated Membranes—Since monoclonal antibody 10E5 binds to the associated GPIIb-GPIIIa complex, not to the EDTA-dissociated complex (24), it was employed to obtain further proof that neuraminidase could reassociate the EGTA-dissociated complex. CIE plates of platelet membranes dissociated with EGTA and reassociated with neuraminidase were therefore incubated with ¹²⁵I-labeled F(ab')₂ fragments.
Effect of Charge on GPIIb-GPIIIa Complex

**FIG. 6.** Binding of $^{125}$I-fibrinogen to the associated, EGTA-dissociated, neuraminidase-reassociated, and neuraminidase-treated major antigen complex of the Triton-solubilized platelet membrane. *a,* 50 µg of control platelet membranes; *b,* membranes incubated with 1 mM EGTA for 5 min; *c,* membranes incubated with 0.1 unit of neuraminidase for 20 min, followed by buffer for 5 min; *d,* membranes incubated with 1 mM EGTA for 5 min, followed by 0.1 unit of neuraminidase/50 µg of membrane for 20 min. The washed, blotted CIE slides were then incubated with $^{125}$I-fibrinogen (100,000 cpm/ml) overnight, followed by washing, blotting, and drying prior to radioautography. Autoradiograms of slides *e-h* refer to their respective slides *a-d,* which have been stained with Coomassie Brilliant Blue.

**FIG. 7.** Effect of the cationic peptide tetralysine on reassociation of the EGTA-dissociated complex of the Triton-solubilized platelet membrane. *a,* 50 µg of control platelet membranes; *b,* membranes incubated with 1 mM EGTA for 5 min at room temperature, followed by CIE buffer for 20 min; *c,* membranes incubated with 1 mM EGTA for 5 min, followed by 5 mM tetralysine for 20 min.

Neuraminidase Treatment of Washed Platelets—Fig. 3, *a--d,* demonstrates the effect of neuraminidase treatment of washed platelets prior to the preparation of platelet membranes in the usual fashion. Fig. 3a represents membrane from washed platelets treated with buffer. Fig. 3, *b--d,* represents membrane from washed platelets treated with neuraminidase. Dissociation of the complex was not impeded following addition of EGTA (Fig. 3b), nor was reassociation of the complex impeded following the addition of Ca$^{2+}$ (Fig. 3d). Furthermore, partial reassociation of the EGTA-dissociated complex could be accomplished by treating these extracted solubilized membranes with neuraminidase (Fig. 3c). Note the cathodal shift of the GPIIb-GPIIIa complex (Fig. 3, *b--d,* compared to Fig. 3a), as well as the shortening and widening of the fully associated complex (Fig. 3d) as in Fig. 1d, indicating that negatively charged sialic acid residues had been removed from the complex on the cell surface.
Effect of Charge on GPIIb-GPIIa Complex

Fig. 4, a–d, demonstrates the effect of neuraminidase treatment of washed platelets in the presence of 20 μM ADP. Following ADP and neuraminidase treatment, the associated complex of the Triton-solubilized platelet membrane extract dissociates minimally in the presence of EGTA. Compare Fig. 4, a and b (pretreatment of platelets with ADP alone), in which 86% of the complex is dissociated with EGTA (calculated from the areas subtended by the precipitin arcs of the GPIIb-GPIIa complex) with Fig. 4, c and d (pretreatment with ADP and neuraminidase), in which 30% of the complex is dissociated with EGTA. Similar results were obtained in three other experiments, with dissociation in the absence of neuraminidase treatment of 100, 88, and 60% compared to dissociation following ADP and neuraminidase treatment of 8, 29, and 23%, respectively. These data indicate that ADP has perturbed the intact platelet membrane, allowing more sialic acid residues to become accessible to neuraminidase treatment.

4Ca²⁺ Binding to Neuraminidase-treated Membranes—Fig. 5, a–h demonstrates the effect of Ca²⁺ binding to the associated major antigen GPIIb-GPIIa complex, the EGTA-dissociated complex, the neuraminidase-reassociated complex in the absence of Ca²⁺, and the associated complex, which had been treated with neuraminidase. Ca²⁺ binds to the associated complex (Fig. 5, a, e), to GPIIb of the EGTA-dissociated complex (Fig. 5, b, f), to the EGTA-dissociated complex reassociated with neuraminidase (Fig. 5, c, g), and to the associated complex which had been treated with neuraminidase (Fig. 5, d, h).

¹²⁵I-Fibrinogen Binding to Neuraminidase-treated Membranes—Fig. 6 demonstrates that ¹²⁵I-fibrinogen binds to the associated complex (Fig. 6, a, e), the EGTA-dissociated complex reassociated with neuraminidase (Fig. 6, d, h), and the associated complex which had been treated with neuraminidase (Fig. 6, c, g). ¹²⁵I-Fibrinogen did not bind to either GPIIb or GPIIa of the EGTA-dissociated complex (Fig. 6 b, f). ¹²⁵I-Fibrinogen also binds to fibrinogen as well as a cathodal arc with the same mobility and characteristics as thrombospondin (13, 25). Similar results were obtained after preincubating the plates with 0.05% bovine serum albumin, by washing with albumin prior to the addition of ¹²⁵I-fibrinogen. Preincubation with nonradioactive fibrinogen at 10–100 times the concentration of ¹²⁵I-fibrinogen completely blocked binding of radioactively labeled fibrinogen in the immunoprecipitate arcs.

Cationic Peptides—Since removal of negative charges allowed the EGTA-dissociated complex to associate in the absence of free Ca²⁺, it was postulated that other agents affecting the surface charge might also associate the complex. Such proved to be the case with tetrylasine, a cationic peptide with pI of 10.5 (Fig. 7, a–c). Note complete association of the EGTA-dissociated complex at 5 mM tetrylasine (Fig. 7c). Partial association was noted with 5 mM arginylarginine, pI 11.3, data not shown.

DISCUSSION

The technique of crossed immunoelectrophoresis to describe the association of GPIIb and GPIIa in the absence of Ca²⁺ by the removal of negatively charged sialic acid residues with neuraminidase, or addition of cationic peptides.

Of particular interest was the effect of elastase, neuraminidase, and tetralysine on the EGTA-dissociated complex. These enzymes, as well as the cationic peptide, reassociate the complex in the absence of Ca²⁺—partially for elastase and almost completely for neuraminidase and tetralysine. Neuraminidase was shown to effect GPIIb, since it moved cathodally, whereas GPIIIa remained stationary. Reassociation with neuraminidase rather than co-migration of neuraminidase-treated GPIIb with GPIIIa was verified by employment of monoclonal antibody 10E5, which reacts specifically with the associated complex (24). The ¹²⁵I-labeled antibody was shown to bind to the neuraminidase-associated complex as well as the Ca²⁺-associated complex, but not to GPIIb or GPIIIa of the dissociated complex. An alternative possibility would be that 10E5 reacts with neuraminidase-treated GPIIb, which co-migrates with GPIIIa, and that fibrinogen binds to neuraminidase-treated GPIIb. We consider this unlikely because a residual "nonassociated" GPIIIa arc, separate from the complex, is usually seen with the neuraminidase-associated" GPIIb-GPIIa complex, and fibrinogen has been shown to attach to GPIIIa, not GPIIb (27). Thus, Ca²⁺ does not appear to be necessary for association of the complex or reactivity of the rabbit anti-platelet membrane antibody for the complex. These data are compatible with the removal or neutralization of a negatively charged moiety (containing negatively charged sialic acid residues) from GPIIb, which appears to prevent fibrinogen binding to GPIIIa, not GPIIb (27). Thus, Ca²⁺ does not appear to be necessary for association of the complex or reactivity of the rabbit anti-platelet membrane antibody for the complex.

The effect of neuraminidase is of interest in regard to the observations of Davis et al. (28) and Greenberg et al. (29), who noted increased platelet aggregation following neuraminidase treatment. The effect of the cationic peptide is of interest, since cationic peptides have been shown to induce platelet aggregation (30–32). The effect of neuraminidase was studied further by pretreatment of washed platelets with neuraminidase prior to the extraction and solubilization of membranes. Although this procedure shifted the major antigen complex cathodally, it had no effect on EGTA-mediated dissociation of the complex, which in turn could be reassociated with neuraminidase. However, prior treatment of the intact washed platelet with ADP, made more sialic acid residues accessible to neuraminidase treatment, leading to the inhibition of the solubilized membrane complex from dissociating in the presence of EGTA. Thus, the critical neuraminidase-sensitive components of the dissociated complex are not exposed on the surface of the inactive platelet membrane, but become exposed after platelet membrane activation. It is of interest in this regard that Motamed et al. (33) noted increased platelet surface sialic acid after platelet shape change with ADP.

The technique of CIE enabled us to study the binding of ⁴Ca²⁺ to the intact major antigen GPIIb-GPIIa complex, as well as the EGTA-dissociated components before and after treatment with neuraminidase. ⁴Ca²⁺ binds to the intact complex before and after sialic acid removal (as noted by the cathodal shift of the complex), as well as to the neuraminidase-associated complex, indicating that easily accessible sialic acid residues are not required for Ca²⁺ binding. ⁴Ca²⁺ also binds to GPIIb; however, it does not bind to GPIIIa.

Reassociation of the EGTA-dissociated complex, in the absence of Ca²⁺, enabled us to study the requirement of Ca²⁺ for fibrinogen binding. Fibrinogen bound to the neuraminid-
dase-reassociated complex in the absence of Ca\textsuperscript{2+}, indicating that Ca\textsuperscript{2+} was not required for the binding of fibrinogen to the GPIIb-GPIIIa complex, and that easily accessible sialic acid residues were not required for fibrinogen binding. These studies also indicated that fibrinogen did not bind to the dissociated complex on CIE, confirming the observation of Gogstad et al. (13).

The above data allow the following working hypothesis: Glycoproteins GPIIb and GPIIIa are prevented from associating into their proper conformation for fibrinogen binding by negatively charged sialic acid residues on GPIIb, or negatively charged sialic acid residues and a negatively charged protein domain. The sialic acid residues which maintain the complex in their relatively "dissociated" conformation are not present (or accessible) on the surface of the resting platelet membrane, but become accessible following ADP stimulation or membrane extraction and solubilization. Ca\textsuperscript{2+} binds to GPIIb, and, in so doing, neutralizes negative charges, inducing a conformational change, thus allowing the complex to properly associate for fibrinogen binding. The fibrinogen receptor on the GPIIb-GPIIIa complex does not require Ca\textsuperscript{2+} or sialic acid residues for fibrinogen binding.

REFERENCES